IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants	Sawitzki et al.
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Title	IMMUNE MARKERS USED FOR DIAGNOSIS AND THERAPY IN CONNECTION WITH TRANSPLANT REACTIONS
Art Unit	1634
Examiner	Bausch
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Attorney Docket No.	074060.2

DECLARATION OF BIRGIT SAWITZKI, Ph.D.
UNDER 37 C.F.R. §1.132

I, BIRGIT SAWITZKI declare as follows:

1. I am an inventor on the referenced patent application. My present position is Professor Transplantation Immunology, Department of Transplantation Tolerance, Institute of Medical Immunology, Charité University Medicine Berlin, the assignee of my application. I have over 15 years experience medical immunology, which encompasses my application. My Curriculum Vitae is attached.

2. I have read the October 9, 2009 Office Action and understand, but disagree with, the Examiner's position, as I subsequently explain.

3. I have invented a method to detect graft reactions in a patient. A reaction is either rejection or tolerance. My method determines the level of T8 (SEQ ID NO. 7) in the patient and compares that level to a control. A graft rejection in the patient, or tolerance in the patient (i.e., the absence of a graft reaction), is determined when the patient's level of T8 differs compared to the T8 level in the control.

4. I describe at p. 21 lines 15-18 that T8 (SEQ ID NO. 7) is highly expressed in grafts of tolerance-developing receptor animals. I have described at p. 18 lines 27-29 that T8 is drastically decreased at the time of graft rejection. I thus described that a stably high expression in SEQ ID NO. 7 is predictive of graft acceptance (i.e., tolerance), while a reduced expression of SEQ ID NO. 7 is predictive of graft rejection. I have described at p. 10 lines 19-23 "how much increase or decrease of SEQ ID NO. 7" as a detectable change in the level as compared to the control level. One skilled in this art would recognize that detectable changes in the level of SEQ ID NO. 7 are those changes that can be distinguished from the control level, e.g., based on statistical analysis. Thus, one skilled in this art can practice my method by determining that a detectable increase in the level of SEQ ID NO. 7, compared to a control level, indicates

graft tolerance, and that a detectable decrease in the level of SEQ ID NO. 7, compared to a control level, indicates graft rejection.

5. Each of my FIGS. 2-4 show error bars of a statistical analysis, demonstrating that a number of animals were analyzed. I have also described statistically relevant results for a number of animal models and a number of different transplants. My kidney transplantation data show the results of syngen (n = 5), allogen (non-treated; n = 5), and allogen (anti-CD4 treated; n = 5) animals. My heart transplantation data show the results of Rag -/- (n = 3), allogen (non-treated; n = 3), and allogen (anti-CD4+DST treated; n = 3) animals. My liver transplantation data show the results of n = 3 animals. My data were analyzed using the statistical software SPSS (SPSS GmbHSoftware, Munich Germany) and are reported as mean ± SD. Data for gene expression between treatment groups, (e.g. between a control group and a treatment group) were analyzed by the Friedmann test, followed by using the MWU test for pair-wise comparison. Differences were considered significant when $p < 0.05$. I also submit my published data, American Journal of Transplantation, 7 (2007) 1091, which I have attached as Exhibit A, indicating acceptance by the scientific community.

6. My method recites that modified levels of SEQ ID NO. 7, as compared to control levels, are indicative of graft reaction. My method recites the use of functional analogues of SEQ ID NO. 7, which are described, e.g., as homologues in other organisms, including human. In addition, and as the Examiner has acknowledged, one skilled in the art routinely identifies homologous mRNA in related species. As I show in Example 5, human homologues of my described sequences, including SEQ ID NO. 7, have been identified, and several of the described sequences are similarly regulated in human patients similar to animal models; I would thus compare the level of the nucleic acid with a control, and determine if there were detectable changes.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both, under § 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the subject application or any patent issued thereon.

24.2.2010
Date

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Identification of Gene Markers for the Prediction of Allograft Rejection or Permanent Acceptance

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The clinical success of new treatment strategies aiming on inducing permanent graft acceptance will rely on the ability to determine whether specific unresponsiveness to donor alloantigens has developed and for how long it is maintained. To identify markers for such posttransplant monitoring, genes differentially expressed by graft infiltrating leukocytes during tolerance induction or rejection after kidney transplantation in rats were compared. A subsequently performed full kinetic analysis in two different transplant models, kidney and heart, in two species, rat and mouse identified two markers (TOAG-1, α-1,2-mannosidase) with high specificity and reproducibility, which are highly expressed during induction and maintenance of acceptance, and downregulated during rejection. Expression level of these markers showed a strong positive correlation with graft function. In addition, expression of both genes was downregulated in the peripheral blood and the graft prior to rejection, suggesting that these markers may be useful for monitoring in clinical transplantation where peripheral blood is the most easily accessible patient sample. Interestingly, downregulation of TOAG-1 and α-1,2-mannosidase expression occurred in graft infiltrating cells and expression of both genes was also downregulated after T-cell activation *in vitro*.

Key words: Gene expression, rejection, T cell, tolerance, transplantation

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Introduction

Achieving long-term, drug-free graft acceptance is still an unsolved problem in clinical transplantation (1). Several protocols such as mixed chimerism, Campath-1 and anti-CD3 designed with the objective of inducing permanent acceptance are currently being investigated in clinical studies in transplantation and autoimmune diseases (2–12). The successful translation of these experimental protocols to the clinic still faces a number of challenges, including the potential impact of memory T cells, heterologous immunity (coincident infections) and homeostatic proliferation (13–15). It is therefore critical that the immune status of the recipient can be monitored before and after treatment to determine when specific unresponsiveness to donor alloantigens or self antigens develops, for how long it is maintained and when a patient is at risk of rejection.

In transplantation, identifying gene markers, whose expression within the graft, fluids draining from the graft, for example, urine or lavage fluid, and ideally the peripheral blood correlates with either long-term graft function or rejection may be one way of monitoring the success or failure of a tolerance induction therapy or immunosuppression minimization strategy. Many investigators have attempted to identify markers of immune function to help diagnose acute rejection or the onset of chronic rejection and to distinguish them from other disorders (16–18). Genes expressed by cytotoxic T cells such as perforin and granzyme B are upregulated within the graft during rejection episodes (19–22) as well as in urine samples during acute and chronic rejection (23). In the peripheral blood perforin mRNA expression was found to predict acute rejection episodes (24). More recently, researchers have applied microarray technology to identify genes whose expression is increased in the graft during acute rejection of mouse hearts (25) and human renal allografts (26–28), and to predict the development of chronic renal allograft rejection (29). Furthermore, Deng et al. have identified a set of genes whose expression analysis allows a noninvasive discrimination (analysis of peripheral blood mononuclear cells) of rejection in cardiac allograft recipients (30). Hoffmann et al. have studied the gene expression in biopsies from normal kidneys, stable kidney allografts, allografts with subclinical rejection and clinical rejection. They identified T bet, FasL and CD152 as good markers to distinguish the above-mentioned patient categories (31). Whether the markers can be used to monitor induction and maintenance of tolerance or permanent acceptance is as yet unknown.

EXHIBIT A

Here we report the identification of new markers whose expression is associated with long-term graft acceptance (TOAG-1, α-1,2-mannosidase). The expression pattern of these gene fragments was found to be consistent in two different transplant models, kidney and heart, and to replicate across two species. Furthermore, the expression of TOAG-1 and α-1,2-mannosidase was not only downregulated in the graft but also in peripheral blood leucocytes 3 days before acute rejection. Furthermore, an association with high peripheral TOAG-1 transcription with T-cell–mediated regulation could also be detected in an experimental autoimmune model. Taken together, these data demonstrate the identification of markers for post-transplant monitoring of graft acceptance or rejection.

Material and Methods

Animals

Male inbred DA ($RT1^{av1}$) and Lewis ($RT1^{l}$) rats (weight 250–300 g) were purchased from Harlan-Winkelmann GmbH (Borchen, Germany).

C57BL/6 (BL6; $H2^{b}$) and C57BL/6Rag1(-/-)(BL6Rag; $H2^{b}$) were purchased from Berliner Institut für Risikobewertung (Berlin, Germany).

CBA/Ca (CBA;$H2^{k}$) and C57BL/10 (B10;$H2^{b}$) were originally purchased from Harlan (Bicester, UK). All mice were bred and housed in the SPF facilities of the Biomedical Services Unit, John Radcliffe Hospital (Oxford, UK). All donor and recipient mice were sex- and age-matched, between 8 and 12 weeks, at the time of transplantation were treated in strict accordance with the Animals (scientific procedures) Act of 1986.

Grafting techniques

Rat kidney transplantation: DA ($RT1^{av1}$) donor kidneys were transplanted to Lewis ($RT1^{l}$) recipients as previously described (32). For the induction of permanent graft acceptance rats were treated with either the anti-CD4 mAb (RIB5/2) at a high dose of 20 mg/kg b.w./day intraperitoneally on days −1, 0, +1, +2, +3 posttransplantation (Tx). Treatment of kidney graft recipients with a control antibody does not lead to prolongation of graft survival with a mean survival time of 6.2 ± 0.4 days. Application of only 5 × 2.5 mg/kg b.w./day anti-CD4 antibody intraperitoneally or 10 × 0.5 mg/kg b.w./day Cyclosporin A subcutaneously does also not lead to a significant prolongation of graft survival with mean survival times of 8.3 ± 1.1 and 7.6 ± 0.7, respectively.

Mouse heart transplantation: B10 ($H2^{b}$) donor hearts were transplanted into CBA mice ($H2^{k}$) was performed essentially as described by Corry et al. (33).

For the induction of permanent graft acceptance (34) naïve CBA ($H2^{k}$) mice were pretreated with YTS177.9 anti-CD4 mAb (kindly provided by Professor Herman Waldmann [Sir William Dunn School of Pathology, Oxford, UK] on days −28/27 (200 µg i.v.) together with a B10 ($H2^{b}$) blood transfusion (250 µL whole blood i.v.) [177/B10DST] 27 days before transplantation of a heterotopic B10 heart graft on day 0.

Murine colitis

BL6Rag mice were injected i.v. with 5×10^{5} syngeneic $CD4^{+}CD45RB^{high}$ ± $CD4^{+}CD25^{+}$ T cells from BL6 mice. Mice receiving only $CD45RB^{high}$ T cells developed clinical sings of colitis 4 weeks post-transfer. For RNA analysis 0.5 mL whole blood was drawn into Paxgene tubes (Qiagen) 1 h (day 0) or 7 days after T-cell transfer. Mice were observed daily and weighed weekly. Any mice showing clinical signs of severe disease were sacrificed.

Differential display RT-PCR and PCR select

Differential display RT-PCR: Total RNA was prepared from graft infiltrating leukocytes (GICs) isolated 5 days after transplantation of a WF kidney into a BDIX recipient treated with either anti-CD4 mAb or control mAb. RNA samples were used for differential display analysis as previously described (32).

PCR select: One microgram poly-A RNA (QuickPrep™mRNA Purification Kit, Pharmacia, Uppsala, Sweden) was prepared from GICs collected on day 5 post-Tx from control or anti-CD4 antibody (RIB5/2)-treated kidney transplant recipients. PCR Select was then performed according to manufacturer's instructions (PCR-Select™ cDNA Subtraction Kit, CLONTECH, Palo Alto, CA). cDNA fragments which had been enriched using PCR Select were cloned using the TA cloning kit (Invitrogen, Leek, The Netherlands).

qPCR

For gene expression analysis grafts and blood samples were harvested at the indicated time points. Total RNA was prepared using the Miniprep Kit (Stratagene, Heidelberg, Germany) and reverse transcribed into cDNA by the murine leukemia reverse transcriptase (Gibco BRL, Gaithersburg, MD). QRT-PCR was then performed as previously described (35). Reactions were run using the Model 7700 Sequence Detector (TaqMan™, Perkin-Elmer Applied Biosystems, Rodgau-Jügesheim, Germany). In the case of rat samples, β-*actin* was used as a housekeeping gene. For the evaluation of mouse samples, HPRT was used as a housekeeping gene. The sequences of the oligonucleotides used are displayed in Table 1. Sense and antisense oligonucleotides were purchased from Metabion (Munich, Germany) Probes were obtained from Eurogentec (Cologne, Germany). The rat CD69 qPCR panel was purchased from Applied Biosystems assay no. Rn01459575_m1.

Histology

Transplanted hearts and kidneys were removed, embedded in Tissue-Tek (Miles Laboratories, Elkhart, IN) and snap-frozen in liquid nitrogen. Cryostat sections (7 µm) were air dried, fixed in acetone, and stained with hematoxylin / eosin (HE), Weigert's elastin/van Gieson stain (EVG) or Elastica Doma (EL-DO).

Isolation of graft infiltrating cells

Graft infiltrating leukocytes (GICs) were isolated from kidney and heart grafts by pressing each diced organ through a sieve followed by enzymatic digestion with 0.02% collagenase IV and 0.002% DNase I (Sigma) in PBS for 30 min at 37°C. Lymphoid cells were separated from the tissue suspension using a Ficoll gradient (Amersham, Uppsala, Sweden). $CD4^{+}$ GICs were obtained by flow cytometry using a FACSVantage (BD Biosciences, San Jose, CA) after staining the total GICs isolated with RM4-5 (anti-CD4)-cychrome (BD Biosciences) in PBS/2% FCS for 20 min at 4°C.

In vitro *stimulation*

Single cell suspensions from spleen of naïve CBA mice were prepared in PBS supplemented with 2% FCS (PBS/FCS) (P.A.A. Laboratories GmBH, Linz, Austria). $CD4^{+}$, $CD8^{+}$, $CD11c^{+}$ and $CD11b^{+}$ splenocytes were obtained using the FACSVantage after staining the cell suspensions with RM4-5 (cychrome), 53-6.7 (PE), N418 (FITC) and M1/70 (PE), respectively. $CD4^{+}$ and $CD8^{+}$ T cells were stimulated *in vitro* with 10 µg/mL plate bound anti-CD3 (145-2C11) / anti-CD28 (37.51) antibodies. $CD11c^{+}$ and $CD11b^{+}$ cells were stimulated with 100 µg/mL LPS. $CD11c^{+}$ and $CD11b^{+}$ cells were stimulated for 6 and 12 h, whereas T cells were stimulated for 12, 24 and 48 h. QPCR of TOAG-1 and α-1,2-mannosidase was performed on samples taken prior and after cell stimulation. $CD4^{+}$ T cells from human PBMC's were incubated with the

Table 1: Oligonucleotides used to quantify mRNA expression by qPCR

Name	Rat qPCR primer	Mouse qPCR primer
TOAG-1	fw 5′-CCCGCCCTCAGAGTCTGAGT-3′ rev 5′-CCGAGAGGGCTGGGATATTAAA-3′ probe 5′-TGATCCTCAGCAGGTATGCACCAAGCTTG-3′	fw 5′-CCTTCTACAACCAGCTGCTGAGA-3′ rev 5′-AATGCCCGAGTTCATGCAAG-3′ probe 5′-TGATCCTTAGCAGTGACAGGTATGCGCC-3′
αMan	fw 5′-TCTGACCCATGATCCCAAGTACA-3′ rev 5′-CGTCATAACTCTCATGGGCAATG-3′ probe 5′-TTTCTAGGGCCTCTACGGCTTCCCAGG-3′	fw 5′-CACGACCCCAAGTACAGGACC-3′ rev 5′-CCTGAGTAGCCTCCGTTCACTCT-3′ probe 5′-TTTCTAGAGCCTCCACGGCTTCCCAGG-3′
ß-actin	fw 5′-GTACAACCTCCTTGCAGCTCCT-3′ rev 5′-TTGTCGACGACGAGCGC-3′ probe 5′-CGCCACCAGTTCGCCATGGAT-3′	
HPRT		fw 5′-ATCATTATGCCGAGGATTTGGAA-3′ rev 5′-TTGAGCACACAGAGGGCCA-3′ probe 5′-TGGACAGGACTGAAAGACTTGCTCGAGATG-3′
CD3	fw 5′-CAAAGAAACTAACATGGAGCAGGG-3′ rev 5′-CTTTTTGCTGGGCCATGGT-3′ probe 5′-AGGTTTGGCTGGCCTCTTCCTGGTG-3′	fw 5′-ATTGCGGGACAGGATGGAG-3′ rev 5′-CTTGGAGATGGCTGTACTGGTCA-3′ probe 5′-TCGCCAGTCAAGAGCTTCAGACAAGCA-3′
Perforin	fw 5′-GGTGGAGTGGAGGCTTTTGTG-3′ rev 5′-CCGAGAAGGCCCATCAGG-3′ probe 5′-CCAGGCGAAAACTGTACATGCGACACT-3′	
Foxp3	fw 5′-TGGCAAACGGAGTCTGCAA-3′ rev 5′-TCTCATCCAAGAGGTGATCTGCTT-3′ probe 5′-AGCCGGGAGAGTTTCTCAAGCACTGC-3′	
CD69	ABI	fw 5′-GTTAATAGTGGTCCTCATCACGTCCT-3′ rev 5′-CCAACTTCTCGTACAAGCCTGG-3′ probe 5′-TTGCCTTAAATGTGGGCAAGTACAATTGCC-3′

α-1,2-mannosidase inhibitor kifunensine 2 μg/mL for 24 h and stimulated with CD3 depleted (Cellsystems) PBMC's of a HLA mismatched donor. Twenty-four hours after stimulation, IL-2 production was measured by ELISA (BD Biosciences).

Statistics

Data were analyzed using the statistical software SPSS (SPSS GmbHSoftware, München, Germany) and are reported as mean ± SD. Data for gene expression between treatment groups were analyzed by Friedmann test followed by using MWU test for pair wise comparison. Differences were considered significant when $p < 0.05$.

Results

CD3 and Perforin mRNA expression is increased in grafts of rejecting and tolerance developing recipients

Nondepleting anti-rat CD4 antibody RIB5/2 is very powerful in inducing unresponsiveness *in vivo* and *in vitro* (32,36). This experimental model for the induction of operational tolerance to donor alloantigens was used to identify new diagnostic markers. DA ($RT1^{av1}$) donor kidneys were transplanted to Lewis ($RT1^{l}$) recipients as previously described (32).

First the model was used to analyze whether mRNA expression analysis of previously described 'rejection' marker could be used to monitor tolerance induction. Therefore CD3 and Perforin mRNA expression in grafts of rejecting (Co mAb) and tolerance developing recipients (20 mg anti-CD4 mAb) were studied. As shown in Figure 1 high dose anti-CD4 treatment of kidney graft recipients did not prevent CD3 and perforin upregulation after transplantation although the expression was significantly diminished (day 3: CD3 $p = 0.025$, Perforin $p = 0.05$; day 5: CD3 $p = 0.05$, Perforin $p = 0.05$). Similar results were obtained for FasL, T bet and CD152 (data not shown). As CD3 and Perfroin transcription is also increased 20–50-fold in permanently accepted grafts, expression analysis of these markers in biopsies alone may not be sufficient to assess the success of tolerance inducing therapies.

Isolation of differentially expressed gene fragments in leukocytes infiltrating accepted and rejected rat renal allografts 5 days after transplantation

Graft infiltrating leukocytes (GICs) were isolated from high dose anti-CD4-treated and control antibody-treated recipients 5 days after allogeneic rat kidney transplantation (DA to Lewis) and subjected to differential display analysis. We identified 10 genes that were selectively up- or downregulated in GICs from anti-CD4-treated recipients (Table 2). For further analysis we have concentrated on two genes (Rattus norvegicus similar to hypothetical protein DKFZp313N0621 Acc.No. XM_34508, which we termed tolerance-associated gene 1 = TOAG-1; α-1,2-mannosidase = Acc.No. U04301) that were selectively upregulated in GICs from anti-CD4-treated recipients.

Kinetics of gene expression after rat kidney transplantation

The kinetics of mRNA expression of these differentially expressed cDNA fragments was analyzed using qPCR. Both genes were found to be expressed at high levels in naïve

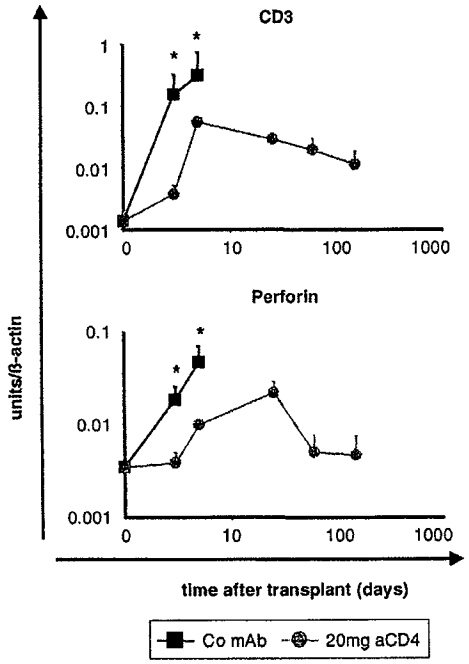

Figure 1: CD3 and Perforin mRNA expression is increased in grafts of rejecting and tolerance developing recipients. Allogeneic kidney transplantation of DA ($RT1^{avl}$) donor kidneys into Lewis ($RT1^{l}$) recipients was performed as described in Material and Methods. CD3 and Perforin mRNA expression in grafts of untreated allograft and high dose anti-CD4 mAb–treated allograft recipients was compared. For tolerance induction 20 mg/kg b.w./day anti-CD4mAb (RIB5/2) was injected intraperitoneally at day −1, 0, +1, +2, +3 post-Tx. Grafts were removed for gene fragment expression analysis on days 2, 5, 25, 60 and 150 after transplantation. Naïve DA kidneys (day 0) were harvested as controls. To analyze gene fragment expression qRT-PCR was performed.

donor rat kidneys (Figure 2). Kinetic analysis revealed that expression of these genes was not downregulated in grafts transplanted into high dose anti-CD4 (20 mg)-treated recipients, whereas expression of both genes was dramatically reduced, several days before rejection occurred, in grafts of control mAb-treated recipients (Figure 2). Importantly, expression of TOAG-1 and α-1,2-mannosidase was always higher in grafts from high dose anti-CD4-treated rat kidney allograft recipients compared with grafts from control mAb-treated recipients (e.g. day 3: TOAG-1 $p = 0.025$, α-1,2-mannosidase $p = 0.034$). Interestingly, a perioperative treatment of kidney graft recipients with a low dose of anti-CD4 antibody or low dose of Cyclosporin A leading only to a slight prolongation of graft survival but no permanent graft acceptance resulted also in intragraft downregulation of TOAG-1 and α-1,2-mannosidase expression (Figure 2, e.g. anti-CD4 20 mg vs. CyA day 3: TOAG-1 $p = 0.05$, α-1,2-mannosidase $p = 0.05$). TOAG-1 expression in nonrejected syngeneic grafts was not downregulated whereas α-1,2-mannosidase transcription was slightly but not significantly reduced (Figure 2, anti-CD4 20 mg vs. syngeneic day 5: TOAG-1 $p = 0.48$, α-1,2-mannosidase $p = 0.3$, Co mAb vs. syngeneic day 5: TOAG-1 $p = 0.021$, α-1,2-mannosidase $p = 0.02$).

Comparative gene expression analysis within the graft after heart transplantation

To validate the data obtained in the rat, we next compared the kinetics of mRNA expression of both genes in mouse heart allografts. α-1,2-Mannosidase and TOAG-1 were again highly expressed in naïve donor mouse hearts. Furthermore, expression of both genes was again reduced several days before rejection occurred (Figure 3). These data are consistent with those obtained in the rat renal allograft model (Figure 2). However, a transient downregulation of the expression of TOAG-1 and α-1,2-mannosidase was also observed in heart allografts transplanted into YTS177/DST pretreated mice. Importantly, the transient decrease of expression in heart allografts transplanted into YTS177/DST pretreated mice was as dramatic as in allografts transplanted into untreated mice, which is in marked contrast to the data obtained after rat renal transplantation (Figure 2).

Long-term accepted heart grafts show signs of ongoing immune activation

Next we investigated the reasons for this difference in expression patterns in the 2 models of graft acceptance. Transcription of CD3 and CD69 in graft from pretreated heart allograft recipients was as high as in grafts from untreated

Table 2: Gene fragments isolated from graft infiltrating cells of renal allografts transplanted into anti-CD4 antibody (acceptance) or control antibody (rejection)-treated rats

	Name	Homology/function	Acc. No.
Acceplance	Kallikrein 7	Serine protease	M19647
Acceplance	Adenylate kinase 4 (AK4)	Nucleotide metabolism	NM_009647
Acceplance	TOAG-1	Unknown	BE115945
Acceplance	α-1,2-mannosidase (Man)	N-glycosylation	U04301
Acceplance	BAP31	Bcl-2 associated protein	XM_215229
Acceplance	CKS1	Cyclin-dependent kinase1	NM_016904
Rejection	EST-2	UNKNOWN	CB785982
Rejection	RHAMM	Receptor for hyaluronic acid–mediated migration	NM_012964
Rejection	RhoGAP	RhoGTPase activating protain	BC024535
Rejection	ATPase II	Chromaffine granule ATPase II	XM_223394

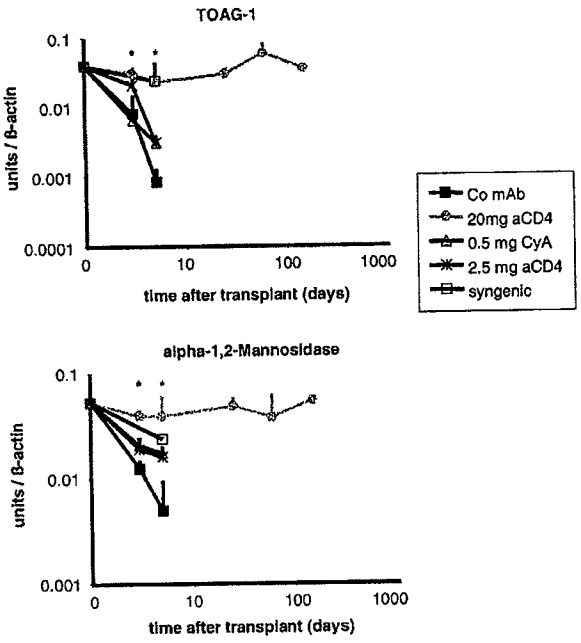

Figure 2: TOAG-1 and α-1,2-mannosidase expression after rat kidney transplantation. Allogeneic and syngeneic kidney transplantation of DA or Lewis donor kidneys into Lewis recipients was performed as described in Material and Methods. Gene fragment expression in grafts of the following groups was compared: untreated kidney allograft recipients (MST = 6.2 ± 0.4), 10 × 0.5 mg/kg b.w./day Cyclosporin A–treated kidney allograft recipients (MST = 7.6 ± 0.7), 5 × 2.5 mg/kg b.w./day anti-CD4 mAb–treated kidney allograft recipients (MST = 8.3 ± 1.1) and 5 × 20 mg/kg b.w./day anti-CD4 mAb–treated kidney allograft recipients (MST > 300). Grafts were removed for gene fragment expression analysis on days 2, 5, 25, 60 and 150 after transplantation. Naïve DA kidneys (day 0) were harvested as controls. To analyze TOAG-1 and α-1,2-mannosidase expression, qRT-PCR was performed. Data are shown as mean ± SD of five independent experiments. *$p < 0.05$

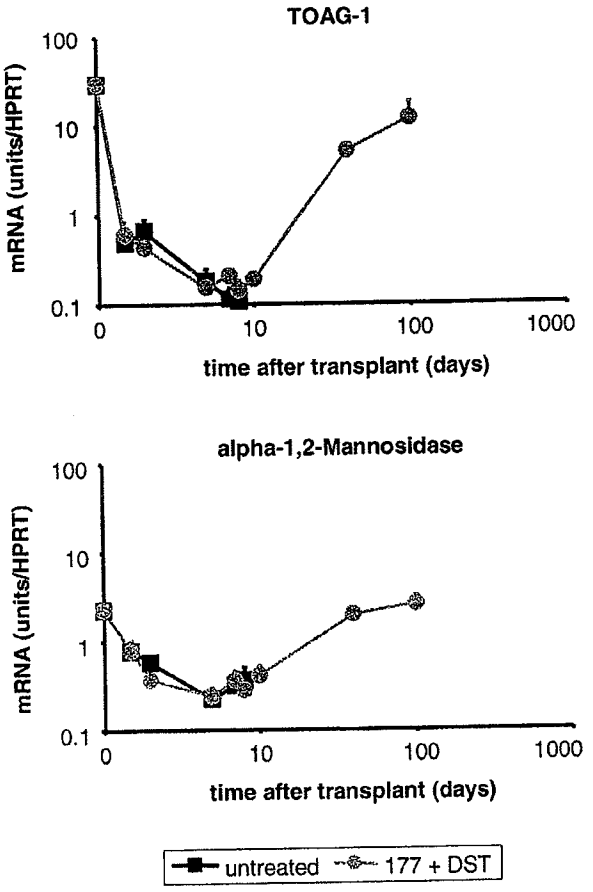

Figure 3: Comparative intragraft gene expression analysis after mouse heart transplantation. Allogeneic heart transplantation of B10 ($H2^b$) donor hearts into CBA ($H2^k$) recipients was performed as described in the Material and Methods. TOAG-1 and α-1,2-mannosidase expression in grafts of the following groups was compared: rejection (untreated allogeneic heart transplants) and acceptance (YTS177/DST pretreated allogeneic mouse heart transplants). Grafts were removed for gene fragment expression analysis on days 1, 2, 5, 7, 8, 10, 40 and 100. B10 hearts were harvested from naïve animals and used as controls (designated day 0). To analyze TOAG-1 and α-1,2-mannosidase expression, qRT-PCR was performed. Data are shown as mean ± SD of three to five independent experiments. *$p < 0.05$

acutely rejecting recipients (Figure 4A). Furthermore, this high expression of CD3 and CD69 was preserved throughout the whole observation period. In contrast, CD3 and CD69 transcription in grafts from high dose anti-CD4 (20 mg) treated kidney allograft recipients although temporary upregulated never reached the magnitude of transcription in grafts from untreated acutely rejecting recipients and was not sustained. These findings therefore suggested that there might be an ongoing immune response within the cardiac allografts of YTS177/DST pretreated mice, such that although the grafts were surviving long term they were subject to an ongoing attack by the host immune system leading to chronic graft dysfunction. To address this possibility, heart grafts were removed for histological analysis 100 days after transplantation. In each case, a proportion of the vessels within the graft showed evidence of luminal occlusion, one of the features of transplant arteriosclerosis (Figure 4B). In contrast, histological examination of long-term surviving kidney grafts harvested 150 days after transplantation revealed no signs of chronic graft dysfunction (Figure 4B). These data are important and interesting as they suggest that down-regulation of TOAG-1 and α-1,2-mannosidase expression within the graft early post-Tx may be useful, not only as markers of acute rejection in the early phase after transplantation but also be indicative of ongoing immune attack to the allograft that could lead to the deterioration of graft function.

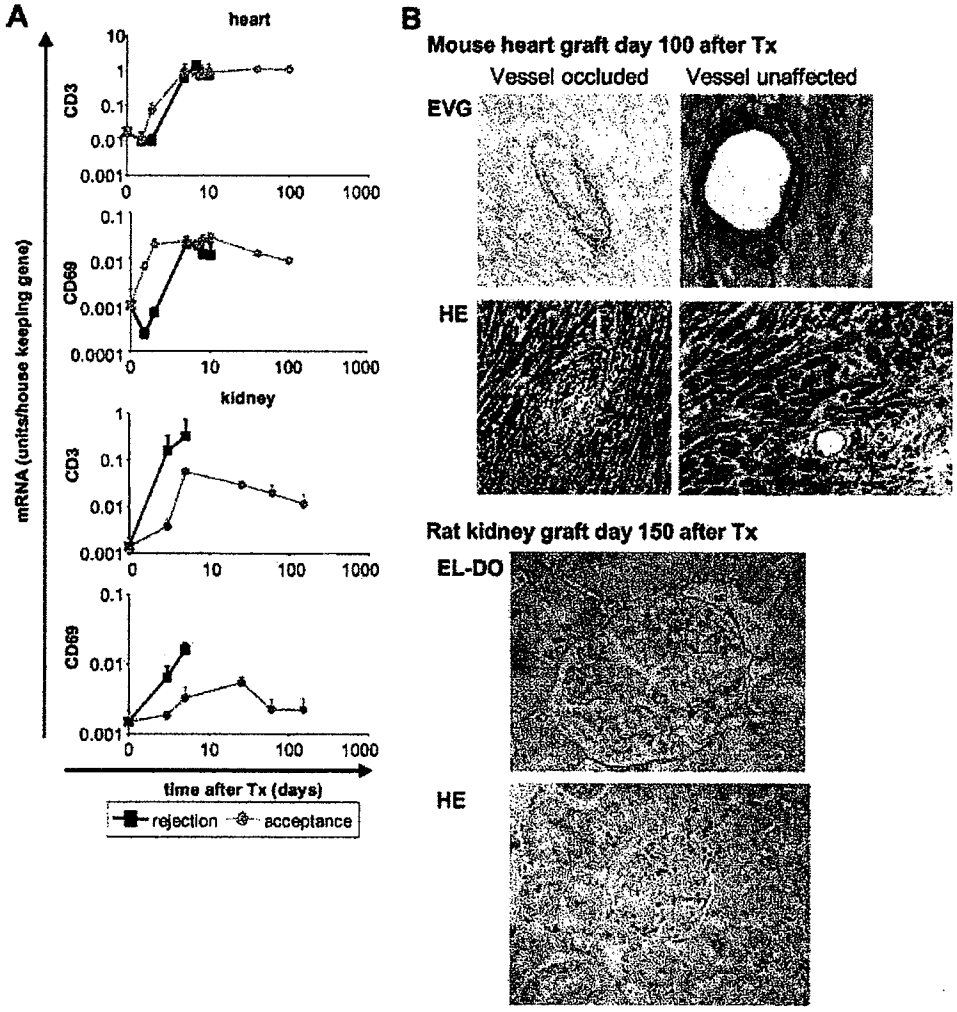

Figure 4: Gene expression and histological analysis after rat kidney and mouse heart transplantation. (A) CD3 and CD69 mRNA expression in the grafts of the following groups was compared: rejection (untreated allogeneic kidney and heart transplants) and acceptance (high dose anti-CD4 mAb–treated allogeneic rat kidney transplants, YTS177/DST pretreated allogeneic mouse heart transplants). Grafts were removed for gene fragment expression analysis on days 2, 5, 25, 60 and 150 after transplantation in case of kidney transplantation, on days 1, 2, 5, 7, 8, 10, 40 and 100 in case of heart transplantation. DA kidneys and B10 hearts were harvested from naïve animals and used as controls (designated day 0). To analyze mRNA expression qRT-PCR was performed. Data are shown as mean ± SD of three to five independent experiments. (B) Representative histological sections of YTS177/DST pretreated heart grafts at day 100 after transplantation were stained with Weigert's elastin / van Gieson (EVG, ×400 original magnification) and hematoxylin/eosin (HE, ×100 original magnification). Representative histological sections of high dose anti-CD4 mAb–treated kidney grafts at day 150 after transplantation were stained with Elastica Doma (EL-DO, ×400 original magnification) and hematoxylin/eosin (HE, ×250 original magnification).

Gene expression in peripheral blood leucocytes

If the gene fragments identified are to be useful for the regular assessment of clinical transplant recipients it is important that similar changes to those detected in the graft (Figures 2–4) are also detectable in the peripheral blood. Therefore gene expression of TOAG-1 and α-1,2-mannosidase was analyzed in peripheral blood leucocyte samples taken serially after mouse heart and rat kidney transplantation. Expression of TOAG-1 and α-1,2-mannosidase was found to be downregulated in the peripheral blood 3 to 5 days before acute rejection of mouse heart and rat kidney allografts (Figure 5; kidney day 3 α-1,2-mannosidase $p = 0.05$, day 5 TOAG-1 $p = 0.025$; heart day 5; TOAG-1 $p = 0.05$; α-1,2-mannosidase $p = 0.05$). In marked contrast, expression of neither gene was downregulated in peripheral blood leucocytes from

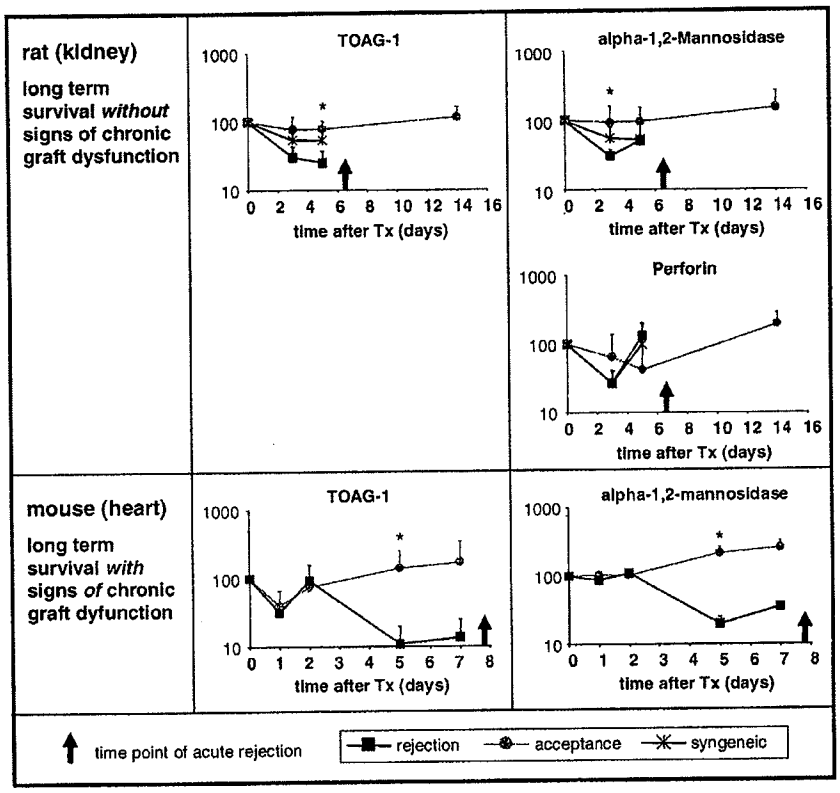

Figure 5: Gene expression in peripheral blood leucocytes of rat kidney and mouse heart graft recipients. Allogeneic and syngeneic kidney transplantation of DA or Lewis donor kidneys into Lewis recipients and allogeneic heart transplantation of B10 donor hearts into CBA recipients was performed as described in the Material and Methods. The following groups were compared: rejection (untreated allogeneic kidney and heart transplants) and acceptance (syngeneic rat kidney transplants, high dose anti-CD4 mAb–treated allogeneic rat kidney transplants, YTS177/DST pretreated allogeneic mouse heart transplants). Recipients were sacrificed and peripheral blood was collected for gene expression analysis at day 0, 3, 5 and 14 after transplantation in case of kidney graft recipients and day 0, 1, 2, 5, 7, 8 and 10 after transplantation in case of heart graft recipients. To analyze gene expression erythrocytes were as described in Material and Methods and qRT-PCR was performed. Data are shown as mean ± SD of three to five independent experiments. *$p < 0.05$

recipients of long-term surviving heart and kidney allografts at the same time point. The decrease in TOAG-1 and α-1,2-mannosidase expression in peripheral blood leukocytes was not due to changes in leukocyte subpopulations (data not shown).

Although we detected a slight downregulation of TOAG-1 and α-1,2-mannosidase transcription in peripheral blood samples of syngeneic kidney graft recipients, this was not significant (Figure 5, anti-CD4 20 mg vs. syngeneic day 3 α-1,2-mannosidase $p = 0.48$, day 5 TOAG-1 $p = 0.18$; rejection vs. syngeneic day 3 α-1,2-mannosidase $p = 0.016$, day 5 TOAG-1 $p = 0.027$).

No significant differences in expression of perforin between peripheral blood samples of acutely rejecting and long-term surviving anti-CD4 (20 mg)-treated allogeneic and syngeneic recipients were detectable early after rat kidney transplantation (Figure 5).

Correlation between high levels of TOAG-1 and α -1,2-mannosidase mRNA expression and the function of long-term accepted heart grafts

Heterotopic heart allografts from YTS177/DST pretreated recipients were palpated at day 55 and scored using a semiquantitative scale by two blinded observers. Grafts were then harvested and intragraft mRNA expression of TOAG-1 and α-1,2-mannosidase analyzed. As illustrated in Figure 6A, expression of both TOAG-1 and α-1,2-mannosidase showed a strong positive correlation with heart graft function (pearson correlation TOAG-1 $R^2 = 0.6163$, $p = 0.007$; α-1,2-mannosidase $R^2 = 0.6157$, $p = 0.007$).

Expression of TOAG-1 and α -1,2-mannosidase is regulated in graft infiltrating cells

In order to determine whether downregulation of TOAG-1 and α-1,2-mannosidase expression occurred only in GICs or also in the parenchyma, mRNA expression was analyzed in (i) whole organ grafts, (ii) isolated GICs and (iii) isolated

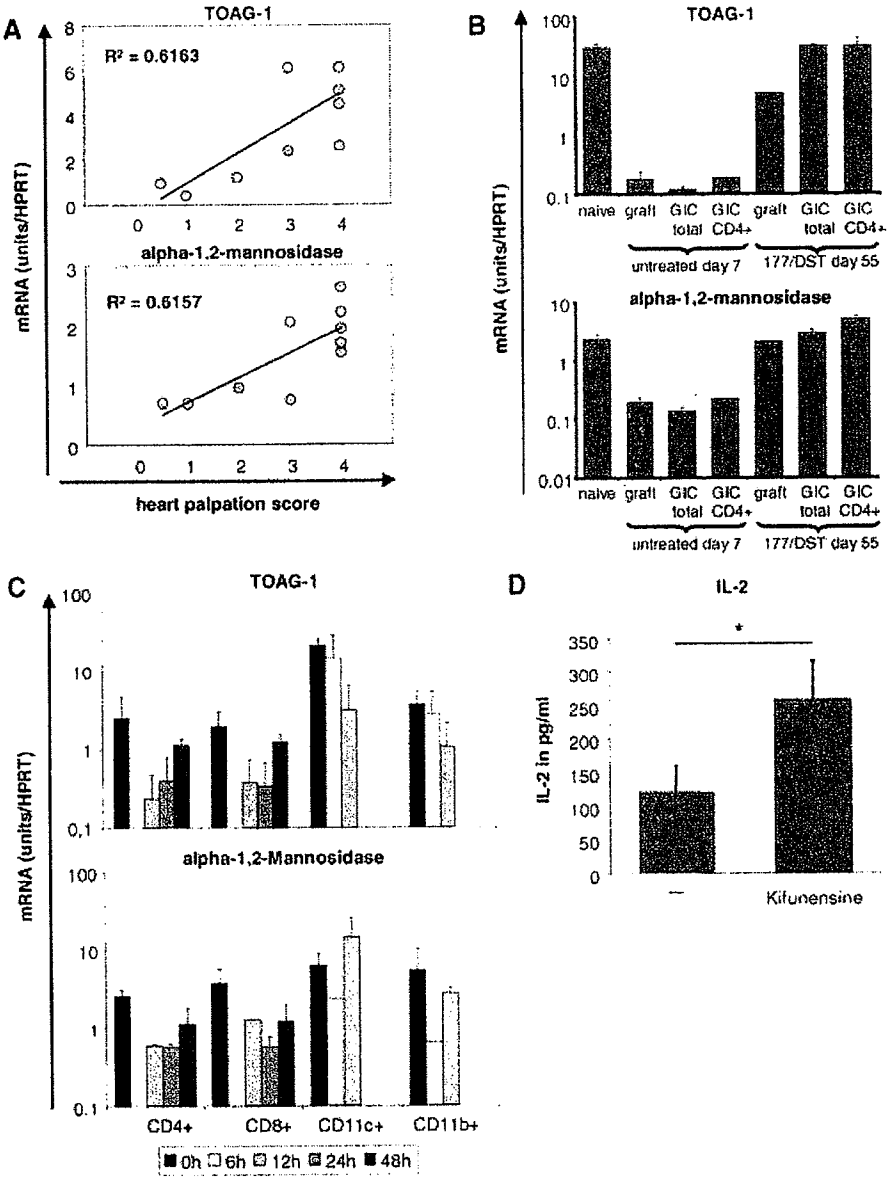

Figure 6: Correlation between high levels of TOAG-1 and α-1,2-mannosidase mRNA expression and the function of long-term accepted heart grafts. (A) TOAG-1 and α-1,2-mannosidase expression in YTS177/DST pretreated allogeneic heart transplants. Hearts transplanted into YTS177/DST pretreated mice were palpated at day 55 and scored on the basis of a semiquantitative scale as described in Material and Methods. Grafts were harvested and intragraft mRNA expression of TOAG-1 and α-1,2-mannosidase analyzed using qRT-PCR and the data obtained correlated with the heart palpation score ($p = 0.007$). (B) TOAG-1 and α-1,2-mannosidase expression by graft infiltrating cells (GICs) isolated from untreated and YTS177/DST pretreated allograft heart graft recipients was analyzed on day 7 and day 55, respectively, by qRT-PCR. GICs were isolated using Ficoll gradient centrifugation. A further isolation of $CD4^+$ T cells (purity > 90%) was achieved by flow cytometry using a FACSVantage. Hearts removed from naïve B10 mice were used as controls. TOAG-1 and α-1,2-mannosidase mRNA expression in naïve hearts, whole organ grafts, total graft infiltrating cells and $CD4^+$ graft infiltrating cells was compared using. Data are shown as mean ± SD of three independent experiments. (C) TOAG-1 and α-1,2-mannosidase expression prior and after *in vitro* activation of individual splenocyte populations. Single cell suspensions from spleen of naïve CBA mice were enriched for CD4, CD8, CD11c and CD11b positive cells using the FACSVantage. $CD4^+$ and $CD8^+$ T cells were stimulated with plate bound anti-CD3 and anti-CD28 antibodies $CD11c^+$ and $CD11b^+$ cells were stimulated with LPS. After the indicated time points cells were harvested and expression of TOAG-1 and α-1,2-mannosidase determined by qPCR. (D) IL-2 production of human $CD4^+$ T cells 24 h after stimulation with HLA mismatched $CD3^+$ depleted PBMC's.

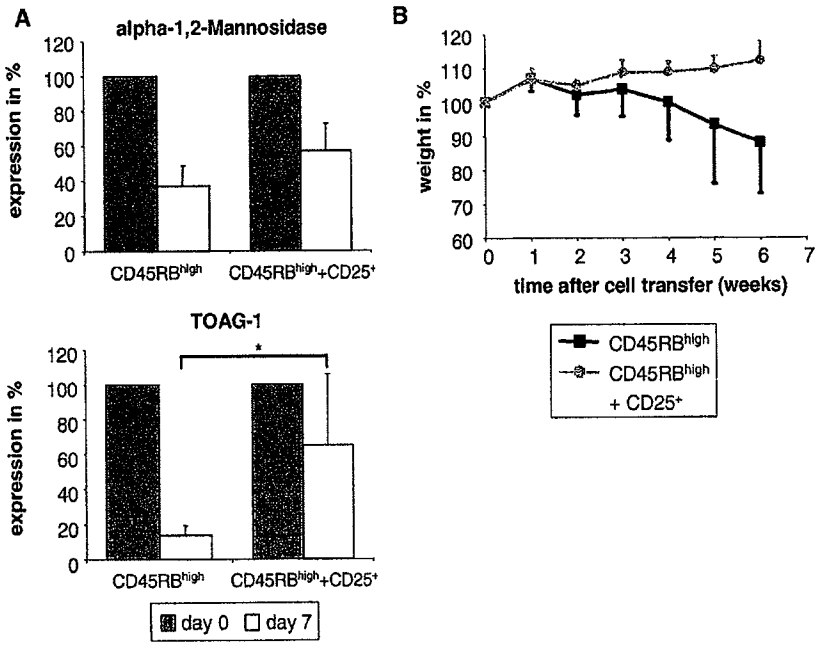

Figure 7: Peripheral expression analysis of TOAG-1 and α-1,2-mannosidase during murine colitis. (A) BL6Rag mice were injected i.v. with 5×10^5 syngeneic $CD4^+CD45RB^{high} \pm CD4^+CD25^+$ T cells from BL6 mice. For RNA analysis 0.5 mL whole blood was drawn into Paxgene tubes (Qiagen) 1 h (day 0) or 7 days after T-cell transfer. To analyze gene expression qRT-PCR was performed. (B) Body weight as percent of initial weight of individual mice. Data are shown as mean ± SD of three to six independent experiments. $^*p < 0.05$.

graft infiltrating $CD4^+$ T cells from untreated, rejecting and YTS177/DST pretreated long-term surviving heart graft recipients. Interestingly, mRNA expression for both genes was significantly reduced in both total and $CD4^+$ GICs isolated from rejecting recipients and was comparable to the level of expression detected in the whole organ grafts (Figure 6B). Thus, a rejection associated activation of residual and infiltrating leucocytes may account for the diminished TOAG-1 and α-1,2-mannosidase expression. Similar results were obtained *in vitro*. Stimulation of $CD4^+$ and $CD8^+$ T cells with anti-CD3 and anti-CD28 antibodies or $CD11c^+$ and $CD11b^+$ cells with LPS resulted in up to a 20-fold decrease in TOAG-1 and α-1,2-mannosidase transcription (Figure 6C) further indicating that downregulation of intragraft TOAG-1 and α-1,2-mannosidase expression is a result of activation of GICs.

Inhibition of α-1,2-mannosidase activity results in enhanced T-cell activation

Next we investigated whether α-1,2-mannosidase activity can influence alloactivation of T cells. Therefore human $CD4^+$ T cells were incubated with or without the α-1,2-mannosidase inhibitor kifunensine for 24 h. After extensive washing, $CD4^+$ cells were stimulated with $CD3^+$ depleted PBMC's of a HLA mismatched donor. Twenty-four hours after stimulation supernatants were harvested and the amount of secreted IL-2 determined. As shown in Figure 6D inhibition of α-1,2-mannosidase activity prior to T-cell activation resulted in a significant increase of IL-2 production suggesting that α-1,2-mannosidase negatively regulates T-cell activation.

High peripheral TOAG-1 expression is associated with T-cell–mediated regulation

In order to test whether high intragraft and peripheral expression of TOAG-1 and α-1,2-mannosidase is related to the tolerant state and that their expression regulation is not only limited to alloactivation of T cells after transplantation but also occurs during abrogation of self-tolerance by overactivation of auto-reactive T cells, we have determined changes in peripheral TOAG-1 and α-1,2-mannosidase expression in a well-accepted murine colitis model. BL6Rag mice were reconstituted with 5×10^5 syngeneic $CD4^+CD45RB^{high} \pm CD4^+CD25^+$ T cells from BL6 mice. Mice receiving only $CD45RB^{high}$ T cells developed clinical signs of colitis 4 weeks post-transfer resulting in weight loss (Figure 7B). Weight loss and histological signs of colitis (data not shown) can be prevented by cotransfer of $CD4^+CD25^+$ regulatory T cells. In peripheral blood samples of mice receiving only $CD45RB^{high}$ T cells a reduction in TOAG-1 and α-1,2-mannosidase expression 7 days post-transfer was detectable (Figure 7A). This reduction was

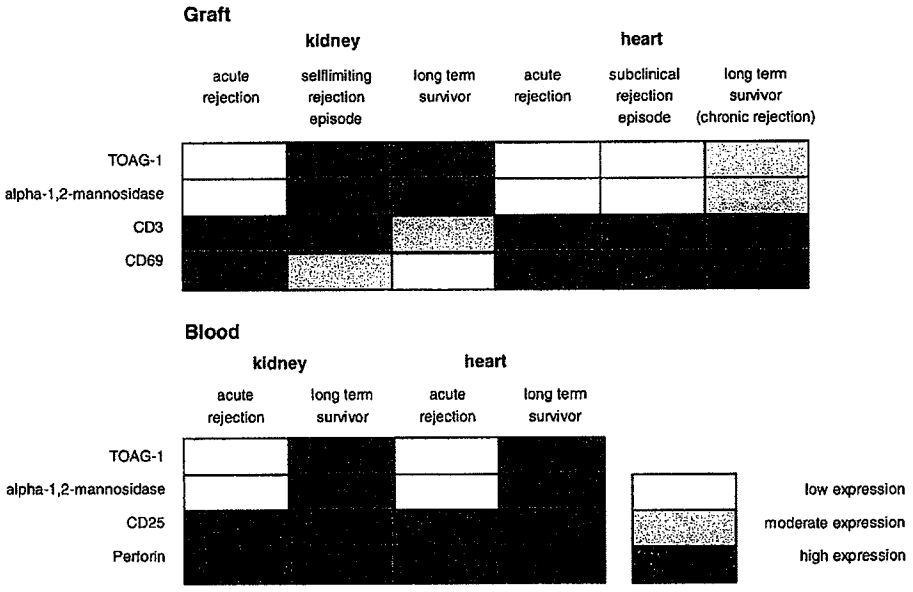

Figure 8: Expression results of the two identified gene markers in kidney and heart grafts as well as PBMC's of kidney and heart graft recipients.

only slightly (α-1,2-mannosidase, $p = 0.101$) or nearly completely (TOAG-1, $p = 0.011$) prevented, when $CD4^+CD25^+$ regulatory T cells were cotransferred. These data indicate that regulation of α-1,2-mannosidase and TOAG-1 expression is not only limited to T-cell activation after transplantation but also occurs during activation of auto-reactive T cells. Furthermore, high levels of TOAG-1 expression seem to be associated with the presence of regulatory T cells preventing excessive T-cell activation.

Discussion

Here we describe the identification of gene markers whose expression in the graft and in the peripheral blood can be used to monitor the success or failure of tolerance induction.

None of the two identified genes TOAG-1 and α-1,2-mannosidase has been described as being important for rejection or permanent acceptance previously. The association of the expression profiles of the two genes with either rejection and/or permanent acceptance was confirmed in two different transplant models in two species by performing a kinetic expression analysis using qRT-PCR; data summarized in Figure 8.

TOAG-1 is identical with the filed sequence 'Rattus norvegicus similar to hypothetical protein DKFZp313N0621, Acc.No. XM'34508'. Although highly conserved among different species, the sequence shows no homology to any known gene and the function of the corresponding protein is therefore unknown. Our preliminary experiments indicate that TOAG-1 is a mitochondrial protein regulating T-cell apoptosis (Gube et al. unpublished observation).

α-1,2-Mannosidase is important for the N-glycosylation of membrane bound and secreted proteins. Inhibition of α-1,2-mannosidase during ConA and anti-CD3 mAb–mediated T-cell activation resulted in increased IL-2 production (37). Our results demonstrate that the magnitude of an alloresponse is higher if α-1,2-mannosidase activity is inhibited in T cells (Figure 6D).

Interestingly, T cells of β-1,6-*N*-acetylglucosaminyltransferase V (Mgat5) deficient mice, an enzyme which is also important for the N-glycosylation of proteins, display a reduced activation threshold due to an enhanced TCR clustering (38). These mice spontaneously develop autoimmune diseases (39). Furthermore, Morgan et al. could demonstrate that *N*-acetylglucosaminyltransferase V (Mgat5)–mediated N-glycosylation negatively regulates Th1 cytokine production by T cells (40).

Thus N-glycosylation of T-cell surface proteins appears to be important for the negative regulation of T-cell activation. Therefore the high expression of α-1,2-mannosidase in graft infiltrating T of long-term surviving grafts may be an important mechanism for the attenuation of alloreactive T-cell responses.

Expression of TOAG-1 and α-1,2-mannosidase was reduced during rejection and high in long-term surviving

grafts. In the long-term surviving kidney grafts from both high dose anit-CD4-treated allogeneic recipients as well as syngeneic recipients no significant transient downregulation of TOAG-1 and α-1,2-mannosidase expression could be detected. In contrast, in long-term surviving heart allografts an early transient downregulation of TOAG-1 and α-1,2-mannosidase expression was observed (Figure 3). This early dramatically reduced TOAG-1 and α-1,2-mannosidase expression was associated with sustained transcription of CD3 and the T-cell activation marker CD69 within the graft and with histological signs of graft vasculopathy (Figure 4). Furthermore, TOAG-1 and α-1,2-mannosidase expression correlated with graft function of long-term surviving heart grafts (Figure 6).

Furthermore, expression of α-1,2-mannosidase and especially TOAG-1 was also regulated in an experimental autoimmune model (Figure 7A). These results further support the importance of both genes for a negative regulation of T-cell activation.

Taken together these findings suggest that expression analysis of these markers in graft biopsies may help to predict acute rejection episodes as well as ongoing chronic allograft dysfunction.

Interestingly, expression of both TOAG-1 and α-1,2-mannosidase was downregulated in the peripheral blood 3–5 days before acute rejection of mouse heart and rat kidney allografts. No significant decrease of TOAG-1 and α-1,2-mannosidase transcription in peripheral blood samples of syngeneic recipients was observed. Thus, expression analysis of these two markers might have potential for detecting acute rejection episodes before clinical signs are apparent.

Recently, several other investigators have studied the gene expression pattern associated with tolerance induction in different transplant models. A set of genes (*TGF-ß2, ppENK, GM2a, GITR, IL-1R2*) were identified by SAGE screening that are specifically upregulated in regulatory T cells associated with tolerance of skin allografts (41,42). Matsui et al. identified two genes (*H2-Ea, Frzb*), which are highly expressed in long-term surviving heart allografts (43). In this model long-term survival was induced by costimulatory blockade. Similar studies were performed by two other groups (44,45). Unfortunately, none of the identified genes overlap with those identified here. Using different transplant models and tolerance induction protocols may explain the missing overlap. In contrast to the above-mentioned studies, the expression pattern of TOAG-1 and α-1,2-mannosidase was validated in different transplant models and in peripheral blood samples.

The data presented here describe the isolation of gene markers whose expression analysis may be useful for monitoring the success or failure of novel strategies for the induction of permanent acceptance to donor alloantigens. Whether peripheral and intragraft TOAG-1 and α-1,2-mannosidase expression can be utilized to monitor success or failure of conventional immunosuppressive therapies has to be investigated. To our knowledge this is the first study performing gene expression profiling after transplantation comparing different models and species.

A direct correlation between the gene expression profile and graft function both in the short and long term was obtained.

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